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The PsbS protein controls the macro-organisation of photosystem II complexes in the grana membranes of higher plant chloroplasts

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ABSTRACT

The PsbS protein is a critical component in the regulation of non-photochemical quenching (NPQ) in higher plant photosynthesis. Electron microscopy and image analysis of grana membrane fragments from wild type and mutant Arabidopsis plants showed that the semi-crystalline domains of photosystem II supercomplexes were identical in the presence and absence of PsbS. However, the frequency of the domains containing crystalline arrays was increased in the absence of PsbS. Conversely, there was a complete absence of such arrays in the membranes of plants containing elevated amounts of this protein. It is proposed that PsbS controls the macro-organisation of the grana membrane, providing an explanation of its role in NPQ.

1. Introduction

Under conditions of excess light, the light harvesting system of plant chloroplasts switches from a state of maximum efficiency of light utilisation to one in which up to 80% of absorbed energy is dissipated as heat [1]. This process is detected as the non-photochemical quenching of chlorophyll fluorescence (NPQ). The major fraction of NPQ is induced rapidly and reversibly in response to the build up of the thylakoid pH gradient ΔpH and is called qE [2]. Although the molecular mechanism of qE has not been proven, there is experimental evidence indicating that the Lhcb-containing light harvesting antenna complexes of photosystem II are involved [1], either confined to one of the minor complexes such as CP26 or CP29 [3] or more diffusely in several complexes, including the major trimeric light harvesting complex of photosystem II (LHCII) [1]. Xanthophylls bound to these complexes play a key role in the quenching mechanism, but there is conflicting evidence concerning whether lutein bound at the internal L1 site [4], or zeaxanthin bound at either the internal L2 site [5] or the peripheral V1 site [6] are involved. In higher plants, the Lhcb-related PsbS protein plays a key role in qE; in *Arabidopsis* mutants lacking this protein, the rapidly forming/relaxing component of NPQ is absent [7], suggesting that qE is either inhibited or dramatically slowed down. Conversely, in plants over-expressing the *psbS* gene and containing an excess of the PsbS protein, the capacity for qE is increased by around twofold [8].

The mechanism by which PsbS exerts such strong control over qE is unclear. It was proposed that it might be the site of quenching, although this now seems unlikely, since PsbS does not appear to stably bind pigments. Reports that PsbS specifically binds zeaxanthin [9] may be explained by its very hydrophobic properties [10] and chlorophyll binding data is also inconsistent [11]. In addition, purified light harvesting complexes readily adopt quenching states [12–14] showing that they possess quenching sites. It has therefore been concluded that the effect of PsbS is an indirect one, in some way controlling the formation of quenchers in the light harvesting complexes [3,15]. A role in sensing the proton gradient is supported by the inhibition of qE in mutants in which specific lumen-facing glutamate residues on PsbS have been eliminated by mutagenesis [16] and by the binding of DCCD to this protein [16]. An alternative, though not necessarily mutually exclusive suggestion, is that PsbS acts as a catalyst of qE formation, aiding the low pH-induced transition of the light harvesting complex into the quenched state [15]. Spectroscopic analyses of

Abbreviations: LHCII, the main light harvesting complex of photosystem II; PSII, photosystem II; NPQ, non-photochemical quenching; qE, the rapidly relaxing component of NPQ dependent upon the thylakoid pH gradient ΔpH

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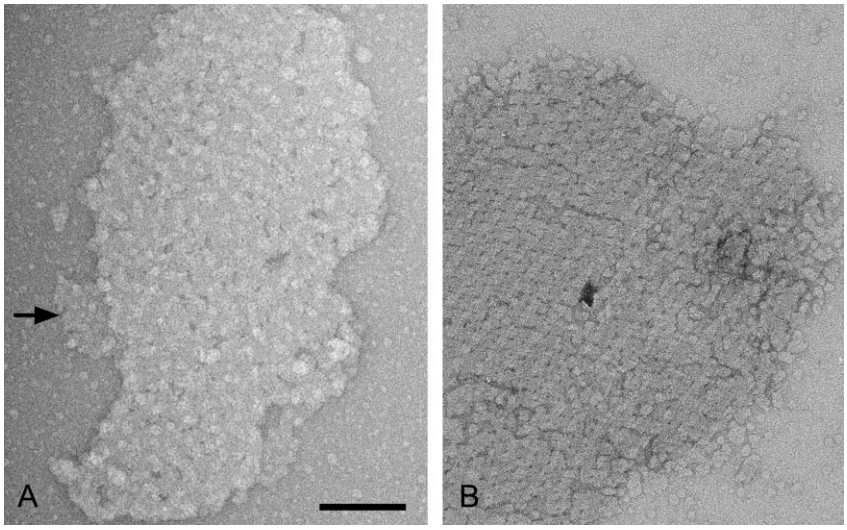

Fig. 1. Examples of images showing non-crystalline (A) and crystalline (B) orientations of photosystem II (PSII) supercomplexes in paired inside out grana membranes, (A) was obtained from the *npq4-1* mutant with a random orientation of PSII supercomplexes. The arrow points to a single piece of membrane, (B) was obtained from WT membrane with an extensive crystalline area, which is actually a superposition of two layers. The membrane part in (B) has been obtained after incubation with a higher amount of detergent than in (A), as can be seen from the circumference, which is less intact in the first. The membrane part in (A) is in a thicker negative stain layer as in (B) leading to a difference in overall contrast. Scale bar is 100 nm for both frames.

purified LHCII in the quenched and unquenched states show that a change in conformation is involved in this transition [17]. These spectroscopic signatures are detected when qE is formed *in vivo* [4]. It was therefore hypothesised that PsbS is involved in this conformational switch [3,4]. It has been argued that qE depends upon the interactions and aggregations between neighbouring proteins in the highly organised macro-structure of the grana membranes [1,18,19]. Here, photosystem II cores are assembled together with antenna complexes to form the LHCII–photosystem II (PSII) supercomplexes [20]. The supercomplexes are sometimes found in highly ordered crystalline arrays, the significance of which is unknown. The complete supercomplex unit, denoted as $C_2S_2M_2$ comprises two PSII cores (C_2), two copies of CP29, CP26 and CP24, plus two strongly bound LHCII trimers (S_2) and a further two trimers bound with moderate strength (M_2). Dissociation of a specific aggregate of CP29, CP24 and the M-LHCII trimer is suggested to be a pre-requisite for qE formation [21].

PsbS is not a component of the purified C_2S_2 complex [22] and can not be accommodated in the image analysis of the $C_2S_2M_2$ complex [20]. This is confirmed by the biochemical analysis of purified $C_2S_2M_2$ supercomplexes [23]. A peripheral location is implied, but it has proved difficult to locate it to any one particular place in membrane fractionation studies, and this has led to the suggestion that it is mobile in the thylakoid membrane [24]. Reversible dimerisation may also determine its location [25]. Thus, could PsbS work by controlling the structure (and therefore the qE function) of PSII supercomplexes? There is already circumstantial evidence for such a view – the reassembly of unstacked thylakoid membranes into grana was found to be controlled by the concentration of PsbS protein [26]. Also, PsbS mutants exhibit defects in PSII electron transport that are not explained by the simple qE-less phenotype [27]. In this paper we describe the results of an investigation in which electron microscopy and image analysis of grana membranes [20] were used to investigate directly the role of PsbS in supercomplex macro-organisation.

2. Materials and methods

Arabidopsis thaliana cv *Columbia* and mutant lines derived from it (*npq4-1* and the PsbS over-expresser *L17*) were grown for 8–9 weeks in plant growth rooms with a 10-h photoperiod at a light intensity of 200 μmol quanta m^{-2} s^{-1} and a day/night temperature of 22/15 °C. Before thylakoid isolation, plants floated on water were pre-treated with 150 μmol photon m^{-2} s^{-1} white light under continuous N_2 and low O_2 for 60 min as described originally by Rees et al. [28]. PSII membrane fragments were separated from thylakoid membranes by FPLC and subject to negative stain electron microscopy as previously described [29]. The xanthophyll compositions of the treated leaves from three replicate experiments were measured by FPLC [30].

3. Results

In these experiments, we wished to observe the grana membrane in a condition that is maximally activated for induction of qE. When dark adapted plants are illuminated, qE forms slowly, because it is limited by the slow rate of de-epoxidation of the xanthophyll cycle pool [30]. In previous work, it has been shown that if leaves are given a pre-illumination period sufficient to activate de-epoxidation, then re-illumination after a short dark relaxation period results in rapid qE formation. A protocol was devised for spinach leaves from which thylakoid membranes were isolated in the de-epoxidised state [28]. This procedure was modified as described in Methods and applied to *Arabidopsis* plants (wild type, the *npq4-1* mutant and *L17*). The de-epoxidation states (DES = ([Zeaxanthin] + 0.5[Antheraxanthin])/([Violaxanthin] + [Antheraxanthin] + [Zeaxanthin]) × 100) of the light treated *npq4-1*, wild type and *L17* plants were 47.89 ± 4.23, 49.38 ± 3.10, and 50.03 ± 2.79, respectively. These leaves, which were maximally poised for qE formation, were used for thylakoid isolation and FPLC allowed separation of PSII-enriched grana membranes. Electron microscopy of the grana membranes reveals the organisation of the PSII supercomplexes; these may be randomly organised (Fig. 1A) or organised into domains of the characteristic crystalline arrays (Fig. 1B), as found previously [31] (Fig. 1B). Similar images were obtained from the thylakoid membranes of both wild type and the *npq4-1* mutant plants.

The crystalline domains were analysed by single particle analysis using GRIP software. They were divided into frames of 76.8 × 76.8 nm, aligned with multireference and non-reference alignment procedures, treated with multivariate statistical analy-

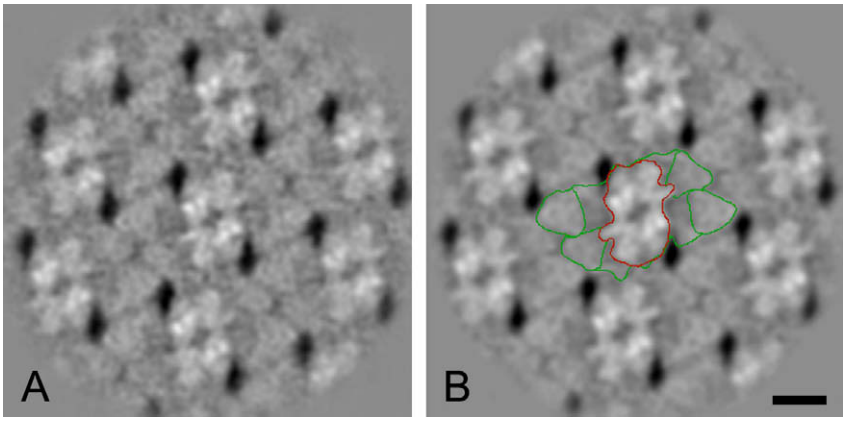

Fig. 2. Final projection maps showing the macro-organisation of photosystem II (PSII) supercomplexes within the grana membranes. (A) Average of 1400 of crystalline domains from the *npq4-1* mutant processed as single particle. (B) Wild type with 1300 crystalline domains as single particle. Twofold symmetry was imposed on both sums. The main light harvesting complex of photosystem II (LHCII)–PSII supercomplex is contoured in green and the PSII core in red. Scale bar is 10 nm.

Table 1
Parameters from electron microscopy and image analysis of photosystem II (PSII) membranes from wild type, *npq4-1* mutant and *L17* over-expresser plants.

Electron microscopy parameter		Plant material		
		Wild type	*npq4-1*	*L17*
Lattice parameter	Dimensions (nm)	27.4 × 21.0	27.8 × 20.2	–
	Angle (deg)	60	62	–
	Area (nm^2)	498.3	495.0	–
Frequency of crystalline domains	Number per 500 micrographs	25	43	0
	Percentage	5.0	8.6	0

Lattice parameters were obtained from the images shown in Fig. 2. The frequency of crystalline domains was determined from the number of electron micrographs showing crystalline arrays as in Fig. 1B. None were detected in the L17 samples, so that no lattice parameters could be calculated. No differences were apparent in the sizes of the domains in wild type and *npq4-1* micrographs.

sis, and classified, as previously described [32]. Averaged images for *npq4-1* and wild type are shown in Fig. 2A and B, respectively. These images show the regular arrays of $C_2S_2M_2$ LHCII–PSII supercomplexes (denoted by the green contour) within which the dimeric PSII core is seen (red). The images in A and B appear identical and indeed numerical values extracted from the images confirm this. The lattice dimensions are very similar (Table 1).

Unlike the case of *npq4-1* and wild type membranes, no crystalline domains were found in the samples prepared from the *L17* over-expresser. Over 500 micrographs were examined. We therefore determined the frequency of micrographs showing these domains in the wild type and *npq4-1* mutant. It was found that the frequency was significantly greater in the *npq4-1* mutant than in the wild type (Table 1).

4. Discussion

The similarity of the lattice dimensions of the crystalline domains in the images of wild type and *npq4-1* mutant grana membranes indicated a near-identical macro-organisation of the photosystem II $C_2S_2M_2$ supercomplexes, irrespective of the presence of PsbS, confirming previous preliminary observations [20]. Clearly, PsbS is *not* a component of the supercomplexes present in these domains. The frequency of the crystalline domains observed was higher in mutant plants without PsbS, compared to the wild type. Similarly, no domains of crystalline arrays were found in any of the micrographs obtained for the samples from the plants with elevated levels of PsbS. This suggests that the content of PsbS determines the organisation of the grana membrane into domains containing crystalline arrays of supercomplexes. In wild type plants, around 5% of micrographs contained these domains, rising to nearly 10% in the absence of PsbS. Such arrays have been observed in whole thylakoids and PSII membranes previously by other methods, such as freeze-fracture electron microscopy (e.g. [33]). These methods indicate that only about 10% of membranes are found in this state, although accurate measurements were not reported. In the present study, we should emphasise that the numerical values of the domain frequencies presented in Table 1 are not exact measurements of either the proportion of membrane in that state or the number of supercomplexes organised into these domains.

The physiological significance of these domains is largely unknown and there have been no previous studies that have indicated that their frequency is dependent on the state or composition of the thylakoid membrane. In the thylakoid membranes, it has been suggested that there is sufficient mobility of complexes in the plane of the membrane to allow a dynamic equilibrium between different kinds of associations between complexes [34]; hence the crystalline arrays and the domains of completely randomly arranged supercomplexes represent two extreme end points of this dynamic equilibrium. Indeed, we have observed areas of membrane in a half-crystalline state, where there are rows of 5–10 supercomplexes, essentially one-dimensional crystals rather than two-dimensional ones. Thermodynamically there is no difference between such crystals, but it was not possible to accurately quantify such "half-crystalline" associations in the present study. According to such a model, in the absence of PsbS, there is a stronger interaction between supercomplexes, which may give rise to the formation of crystals; thus, the effect of PsbS in decreasing the frequency of the domains of crystalline arrays suggests that it weakens this interaction, pulling the dynamic equilibrium away from the tendency to form the crystalline state.

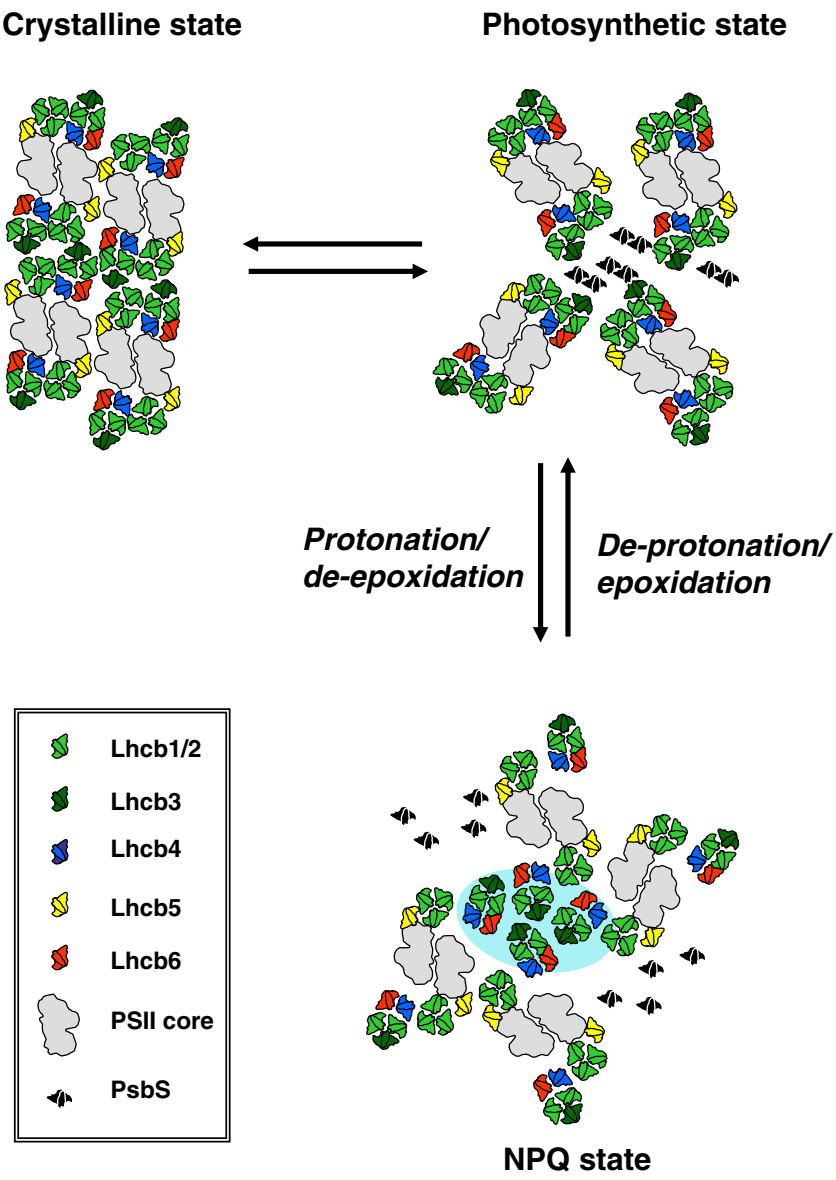

Fig. 3. Model showing how PsbS protein regulates the macro-organisation of photosystem II (PSII) supercomplexes and the induction of the rapidly relaxing component of NPQ dependent upon the thylakoid pH gradient (qE). In this model, modified from [19], there is a dynamic equilibrium that involves three extreme states of organisation of the PSII supercomplexes. The equilibrium between crystalline states and the randomly organised, photosynthetic state is determined by the concentration of dimeric PsbS protein. Only the latter state can rapidly switch to the non-photochemical quenching (NPQ) state to give qE. This switch is driven by protonation and monomerisation of PsbS and regulated by the de-epoxidation of Lhcb-bound violaxanthin to zeaxanthin. Also shown is the putative quenching domain (pale blue), an aggregate of detached CP29, CP24 and M-trimer LHCII as proposed by Betterle et al. [21].

It is interesting to compare this suggested role of PsbS with that of the PufX protein found in purple bacteria. This latter protein controls the long range macro-organisation of the membrane [35] and prevents the formation of ordered crystalline arrays of complexes [36]. PufX-*minus* mutants show defective electron transport [37] and the partial inhibition of PSII electron transfer in the *npq4-1* mutants may therefore have a similar structural basis [27]. Thus, the role of PsbS may be similar to that of PufX, although the effect of its absence is less extreme.

The vital role of PsbS in qE may be explained by these results. We suggest that the changes in conformation and/or interactions between LHCII subunits that are required for the formation of the quenching state require a loose association of PSII supercomplexes. This may also be considered as the fully functional photosynthetic state that is fully competent in electron transport and able to rapidly and reversibly switch between the light harvesting and photoprotective state (Fig. 3). When the tendency for strong interaction is present (in the absence of PsbS), giving rise to an increased frequency of crystalline arrays, these changes in conformation are inhibited and qE is prevented. Specifically, the dissociation of the aggregate of CP29, CP24 and the M-LHCII trimer, which was suggested by Betterle et al. to be an obligatory event in qE formation [21], would be prevented.

The results presented here support the hypothesis that qE involves a PsbS-catalysed re-organisation of PSII supercomplexes in the grana membrane, mediating the transition between the unquenched and quenched states [15]. Whether reversible protonation has any part to play in this mechanism remains to be determined, although the correlation between mutation in lumen-facing glutamate residues on PsbS and both the formation of qE [16] and the dissociation of the PSII supercomplex [21] strongly suggests that this is the case. Certainly, it is possible to conceive of

protonation altering the interaction of PsbS with the PSII supercomplex, and that this is the trigger for qE. Results from Caffari and coworkers [23] show that detergent solubilization has an identical effect on wild type and *npq4-1* mutant membranes, also at low pH when PsbS is protonated and should facilitate the detaching of LHCII from the core [25]. These results thus suggest that the protonation of the two lumenal glutamate residues is not sufficient to activate PsbS and regulate the interactions with LHCII and/or PSII core. Other factors such as a particular ion or the presence of a ΔpH (and not just a low pH as in the in vitro experiment) might be necessary for the activation of PsbS. On the other hand, an alternative explanation is that the primary qE-active protonation trigger resides within the antenna complexes, as indicated previously [38], consistent with the fact that a ΔpH-dependent quenching state can still be induced in *npq4-1* [39], albeit very slowly.

The kinetic heterogeneity, which defines qE and the more slowly relaxing/forming states, qI [40], is explained by the sub-saturating amount of PsbS in wild type plants, associated with a tendency for formation of crystalline domains: where there is a deficiency of PsbS, the NPQ in those domains is of the qI type, since the route towards the rapid development of the quenching state is inhibited. Conversely, where PsbS is saturating, the NPQ is exclusively of the rapid qE type. However, it is important to stress the dual nature of PsbS function first discovered by Kiss et al. [26]. The proposed stabilization of supercomplexes in the photosynthetically active state by PsbS depicted in Fig. 2 is not sufficient for qE – PsbS is also required for the protonation-dependent formation of the quenched state, since the "structural role" of PsbS appears to be fulfilled by proteins mutated at the lumen-facing glutamate residues, which can not catalyse qE formation [26]. Further work is now needed to quantitatively analyse the macro-organisation of grana membranes from a range of mutant plants and from wild type plants grown under a range of conditions, and to correlate this data with the kinetics of NPQ.

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